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PATENT
Attorney Docket No. DIVER1120-1

___ NEW PATENT APPLICATION
___ CONTINUATION-IN-PART
_X_ DIVISIONAL

ASSISTANT COMMISSIONER
FOR PATENTS
Box Patent Application
Washington, D.C. 20231

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Mikhail Bayley
(TYPED OR PRINTED NAME OF PERSON MAILING PAPER OR FEE)

Mikhail Bayley
SIGNATURE OF PERSON MAILING PAPER OR FEE)

Sir:

Transmitted herewith for filing is the divisional patent application of:

Inventors: Dennis Murphy and John Reid

For: **ALPHA-GALACTOSIDASE**

This is a request for filing a ___ continuation _X_ divisional application under 37 C.F.R. 1.53(b), of prior application Serial No. 08/613,220, filed March 8, 1996.

FULL NAME OF FIRST INVENTOR	*LAST NAME:* Murphy	*FIRST NAME:* Dennis	*MIDDLE NAME:*
CITIZENSHIP	*STATE OR FOREIGN COUNTRY:* United States		
POST OFFICE ADDRESS	*POST OFFICE ADDRESS:* 10 Fairway Road	*CITY AND STATE:* Paoli, PA	*ZIP CODE:* 19301
FULL NAME OF SECOND INVENTOR	*LAST NAME:* Reid	*FIRST NAME:* John	*MIDDLE NAME:*
CITIZENSHIP	*STATE OR FOREIGN COUNTRY:* United States		
POST OFFICE ADDRESS	*POST OFFICE ADDRESS:* 922 Montgomery Ave., Apt. J-2	*CITY AND STATE:* Bryn Mawr, PA	*ZIP CODE:* 19010

On page 1, after the title, please insert the following: --This application is a divisional application of U.S. Serial No. 08/613,220, filed March 8, 1996, issuing September 28, 1999 as U.S. Patent No. 5,958,751.--

1. ____ Cancel in this application original claims of the prior application before calculating the filing fee. (At least one original independent claim must be retained for filing purposes.)

2. ____ A preliminary amendment is enclosed.

The filing fee has been calculated as shown below:

For	Number Filed		Number Extra		Rate			Fee	
					Small Entity	Other Entity		Small Entity	Other Entity
Total Claims	10	=	0	X	$9	$0	=	$ .00	$ 0
Independent Claims	2	=	0	X	$39	$0	=	$ .00	0
Multiple Dependent Claims Presented: ___ Yes ___ No					$130	$0			0
BASIC FEE					$380	$760		$380.00	$ 0
					TOTAL FEE			$380.00	

3. _X_ The Assistant Commissioner is hereby authorized to charge a payment of a Total Fee of $380.00 and any other fees associated with this communication or credit any overpayment to Deposit Account No. 07-1895. A duplicate copy of this Transmittal Sheet is enclosed.

_X_ Any additional filing fees required under 37 C.F.R. 1.16.
_X_ Any patent application processing fees under 37 C.F.R. 1.17.

4. _X_ A verified statement claiming small entity status was filed in parent application Serial No. 08/613,220, filed March 8, 1996, and such status is still proper.

5. _X_ The prior application is assigned of record to RECOMBINANT BIOCATALYSIS, INC..

6. _X_ The power of attorney in the prior application is to Lisa A. Haile, Registration No. 38,347.

7. _X_ Please transfer the drawings from the prior application to the new application.

8. _X_ A copy of the prior application as filed is enclosed, including drawings and the Declaration and Power of Attorney filed in parent application, U.S. Serial No. 08/613,220, filed March 8, 1996.

9. _X_ Copies of the Information Disclosure Statements, PTO Forms 1449 and Forms 892 from parent application are enclosed.

10. _X_ Declaration by Inventor(s) (unsigned).

11. _X_ Permission To Use Sequence Listing.

Address all future communications to:

Lisa A. Haile, Ph.D.
GRAY CARY WARE & FREIDENRICH LLP
4365 Executive Drive, Suite 1600
San Diego, California 92121-2189
Telephone: 858-677-1456
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The undersigned states that the enclosed application papers comprise a true copy of the prior application as filed.

Respectfully submitted,

Date: September 28, 1999

Lisa A. Haile, Ph.D.
Attorney for Applicant
Registration No. 38,374

GRAY CARY WARE & FREIDENRICH LLP
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San Diego, CA 92121-2189

C:\CH\APPLTR.FRM D ket No: 331400-40

IN THE UNI[T]ED STATES PATENT AND TRADE[MA]RK OFFICE

Application Transmittal Letter

Box Patent Application
Commissioner of Patents and Trademarks
Washington, D.C. 20231

Dear Sir:

Transmitted herewith for filing is the patent application:

Inventor(s):
Dennis Murphy, and John Reid

For: ALPHA-GALACTOSIDASE

Enclosed with the application are:
1. Specification (25 pages); 2. Claims (2 pages);
3. Abstract; 4. Declaration executed
5. Assignment;
6. Our check number 22518 in the amount of $493.00; and
7. Small Entity Status Form.
8. Two (2) sheets of informal drawings.

The fee has been calculated as follows:

	Number of Claims Filed			Extra Claims	Rate	Fee
Basic Fee					$375	$ 375.00
Total Claims	20	-	20 =	0	x 11	$ .00
Indep. Claims	5	-	3 =	2	x 39	$ 78.00
Recording Fee					$ 40	$ 40.00
TOTAL:						$ 493.00

The Commissioner is authorized to charge payment of any additional filing fees required under 37 CFR 1.16 associated with this communication or credit any overpayment to Deposit Account No. 03-0678.

EXPRESS MAIL CERTIFICATE

Express Mail Label No. _____
Deposit date: March 8, 1996

I hereby certify that this paper and the attachments hereto are being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above addressed to:

Box Patent Application
Assistant Commissioner for Patents
Washington, DC 20231

Charles J. Herron 3/8/96
Charles J. Herron, Esq. **Date**

Respectfully submitted,

Charles J. Herron

Charles J. Herron, Esq.
Reg. No. 28,019
CARELLA, BYRNE BAIN, GILFILLAN,
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VERIFIED STATEMENT(DECLARATION) CLAIMING SMALL ENTITY STATUS

37 CFR 1.9(f) and 1.27(c) - SMALL BUSINESS CONCERN

I hereby declare that I am

[ ] the owner of the small business concern identified below:

[X] an official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF CONCERN: Recombinant BioCatalysis, Inc.

ADDRESS OF CONCERN: 505 Coast Blvd. 4th Floor, La Jolla, CA 92037

I hereby declare that the above identified small business concern qualifies as a small business concern as defined in 13 CFR 121.12, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees under Section 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the number of persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third-party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention entitled:

ALPHA-GALACTOSIDASE

Inventors: Dennis Murphy, and John Reid

described in:
[ ] the specification filed herewith
[X] application serial no. _____, filed _____
[ ] patent no. _____, issued _____

If the rights held by the small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed below and no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under under 37 CFR 1.9(c) if that person made the invention, or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or as a nonprofit organization under 37 CFR 1.9(e).

NOTE: Separate verified statement are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

NAME: _____

ADDRESS: _____

[ ] Individual [ ] Small business [ ] Nonprofit organization

NAME: _____

ADDRESS: _____

[ ] Individual [ ] Small business [ ] Nonprofit organization

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b)).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

NAME OF PERSON SIGNING JAY M SHORT

TITLE IN ORGANIZATION CHIEF TECHNOLOGY OFFICER

ADDRESS OF PERSON SIGNING 320 DELAGE DRIVE, ENCINITAS, CA 92024-4723

SIGNATURE Jay M. Short DATE 3/7/96

ALPHA-GALACTOSIDASE

This invention relates to newly identified polynucleotides, polypeptides encoded by such polynucleotides, the use of such polynucleotides and polypeptides, as well as the production and isolation of such polynucleotides and polypeptides. More particularly, polypeptides of the present invention have been identified as glycosidases and/or α-galactosidases as a result of their enzymatic activity.

In accordance with one aspect of the present invention, there are provided novel enzymes, as well as active fragments, analogs and derivatives thereof.

In accordance with another aspect of the present invention, there are provided isolated nucleic acid molecules encoding the enzymes of the present invention including mRNAs, cDNAs, genomic DNAs as well as active analogs and fragments of such enzymes.

In accordance with another aspect of the present invention there are provided isolated nucleic acid molecules encoding mature polypeptides expressed by the DNA contained in ATCC Deposit No. _____.

In accordance with yet a further aspect of the present invention, there is provided a process for producing such polypeptides by recombinant techniques comprising culturing recombinant prokaryotic and/or eukaryotic host cells, containing a nucleic acid sequence of the present invention, under conditions promoting expression of said enzymes and subsequent recovery of said enzymes.

In accordance with yet a further aspect of the present invention, there is provided a process for utilizing such enzymes for hydrolyzing raffinose (a trisaccharide), converting it to sucrose and galactose. There is application for this enzyme in the beet sugar industry. 20-30% of the domestic U.S. sucrose consumption is sucrose from sugar beets. Raw beet sugar can contain a small amount of raffinose when the sugar beets are stored before processing and rotting begins to set in. Raffinose inhibits the crystallization of sucrose and also constitutes a hidden quantity of sucrose. Thus, there is merit to eliminating raffinose from raw beet sugar. α-galactosidase has also been used as a digestive aid to break down raffinose, stachyose, and verbascose in such foods as beans and other gassy foods.

In accordance with yet a further aspect of the present invention, there are also provided nucleic acid probes comprising nucleic acid molecules of sufficient length to specifically hybridize to a nucleic acid sequence of the present invention.

In accordance with yet a further aspect of the present invention, there is provided a process for utilizing such enzymes, or polynucleotides encoding such enzymes, for *in vitro* purposes related to scientific research, for example, to generate probes for identifying similar sequences which might encode similar enzymes from other organisms by using certain regions, *i.e.*, conserved sequence regions, of the nucleotide sequence.

These and other aspects of the present invention should be apparent to those skilled in the art from the teachings herein.

The following drawings are illustrative of embodiments of the invention and are not meant to limit the scope of the invention as encompassed by the claims.

Figure 1 is an illustration of the full-length DNA and corresponding deduced amino acid sequence of *Thermococcus alcaliphilus* AEDII12RA α-galactosidase 18GC of the present invention. Sequencing was performed using a 378 automated DNA sequencer (Applied Biosystems, Inc.).

The term "gene" means the segment of DNA involved in producing a polypeptide chain; it includes regions preceding and following the coding region (leader and trailer) as well as intervening sequences (introns) between individual coding segments (exons).

A coding sequence is "operably linked to" another coding sequence when RNA polymerase will transcribe the two coding sequences into a single mRNA, which is then translated into a single polypeptide having amino acids derived from both coding sequences. The coding sequences need not be contiguous to one another so long as the expressed sequences ultimately process to produce the desired protein.

"Recombinant" enzymes refer to enzymes produced by recombinant DNA techniques; *i.e.*, produced from cells transformed by an exogenous DNA construct encoding the desired enzyme. "Synthetic" enzymes are those prepared by chemical synthesis.

A DNA "coding sequence of" or a "nucleotide sequence encoding" a particular enzyme, is a DNA sequence which is transcribed and translated into an enzyme when placed under the control of appropriate regulatory sequences.

In accordance with an aspect of the present invention, there are provided isolated nucleic acids (polynucleotides) which encode for the mature enzyme having the deduced amino acid sequence of Figure 1 (SEQ ID NO:4).

In accordance with another aspect of the present invention, there is provided an isolated polynucleotide encoding the enzyme of the present invention. The deposited material is a genomic clone comprising DNA encoding an enzyme of the present invention. As deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852, USA, the deposited material is assigned ATCC Deposit No. _____.

The deposit has been made under the terms of the Budapest Treaty on the International Recognition of the Deposit of Micro-organisms for Purposes of Patent Procedure. The clone will be irrevocably and without restriction or condition released to the public upon the issuance of a patent. This deposit is provided merely as convenience to those of skill in the art and is not an admission that a deposit would be required under 35 U.S.C. §112. The sequence of the polynucleotide contained in the deposited material, as well as the amino acid sequence of the polypeptide encoded thereby, are controlling in the event of any conflict with any description of sequences herein. A license may be required to make, use or sell the deposited material, and no such license is hereby granted.

The polynucleotide of this invention was originally recovered from a genomic gene library derived from *Thermococcus alcaliphilus* AEDII12RA, of the genus *Thermococcus*. AEDII12RA grows optimally at 85°C at pH 9.5.

Accordingly, the polynucleotides and enzymes encoded thereby are identified by the organism from which they were isolated, and are sometimes hereinafter referred to as "AEDII12RA-α-gal-18GC" (Figure 1 and SEQ ID NOS:3 and 4).

The polypeptide of the present invention shows a protein similarity of 52% and protein identity of 21% to *Dictyoglomus thermophilum* amylase.

This invention, in addition to the isolated nucleic acid molecule encoding the enzyme of the present invention, also provides substantially similar sequences. Isolated nucleic acid sequences are substantially similar if: (i) they are capable of hybridizing under conditions hereinafter described, to the polynucleotide of SEQ ID NO:3; (ii) or they encode DNA sequences which are degenerate to the polynucleotides of SEQ ID NO:3. Degenerate DNA sequences encode the amino acid sequence of SEQ ID NO:4, but have variations in the nucleotide coding sequences. As used herein, substantially similar refers to the sequences having similar identity to the sequences of the instant invention. The nucleotide sequences that are substantially the same can be identified by hybridization or by sequence comparison. Enzyme sequences that are substantially the same can be identified by one or more of the following: proteolytic digestion, gel electrophoresis and/or microsequencing.

One means for isolating the nucleic acid molecules encoding the enzyme of the present invention is to probe a gene library with a natural or artificially designed probe using art recognized procedures (see, for example: Current Protocols in Molecular Biology, Ausubel F.M. *et al.* (Eds.) Green Publishing Company Assoc. and John Wiley Interscience, New York, 1989, 1992). It will be appreciated by one skilled in the art that the polynucleotides of SEQ ID NOS:1-2, or fragments thereof (comprising at least 12 contiguous nucleotides), are particularly useful probes. Other particularly useful probes for this purpose are hybridizable fragments of the sequence of SEQ ID NO:3 (*i.e.*, comprising at least 12 contiguous nucleotides).

With respect to nucleic acid sequences which hybridize to specific nucleic acid sequence disclosed herein, hybridization may be carried out under conditions of reduced stringency, medium stringency or even stringent conditions. As an example of oligonucleotide hybridization, a polymer membrane containing immobilized denatured nucleic acids is first prehybridized for 30 minutes at 45°C in a solution consisting of 0.9 M NaCl, 50 mM NaH_2PO_4, pH 7.0, 5.0 mM Na_2EDTA, 0.5% SDS, 10X Denhardt's, and 0.5 mg/mL polyriboadenylic acid. Approximately 2 X 10^7 cpm (specific activity

4-9 X 10^8 cpm/ug) of ^{32}P end-labeled oligonucleotide probe are then added to the solution. After 12-16 hours of incubation, the membrane is washed for 30 minutes at room temperature in 1X SET (150 mM NaCl, 20 mM Tris hydrochloride, pH 7.8, 1 mM Na_2EDTA) containing 0.5% SDS, followed by a 30 minute wash in fresh 1X SET at Tm -10°C for the oligo-nucleotide probe. The membrane is then exposed to auto-radiographic film for detection of hybridization signals.

Stringent conditions means hybridization will occur only if there is at least 90% identity, preferably 95% identity and most preferably at least 97% identity between the sequences. *See* J. Sambrook *et al.*, Molecular Cloning, A Laboratory Manual (2d Ed. 1989) (Cold Spring Harbor Laboratory) which is hereby incorporated by reference in its entirety.

"Identity" as the term is used herein, refers to a polynucleotide sequence which comprises a percentage of the same bases as a reference polynucleotide (SEQ ID NO:3). For example, a polynucleotide which is at least 90% identical to a reference polynucleotide, has polynucleotide bases which are identical in 90% of the bases which make up the reference polynucleotide and may have different bases in 10% of the bases which comprise that polynucleotide sequence. As used herein, a first DNA (RNA) sequence is at least 70% and preferably at least 80% identical to another DNA (RNA) sequence if there is at least 70% and preferably at least a 80% or 90% identity, respectively, between the bases of the first sequence and the bases of the another sequence, when properly aligned with each other, for example when aligned by BLAST.

The present invention relates to polynucleotides which differ from the reference polynucleotide such that the changes are silent changes, for example the change do not alter the amino acid sequence encoded by the polynucleotide. The present invention also relates to nucleotide changes which result in amino acid substitutions, additions, deletions, fusions and truncations in the polypeptide encoded by the reference

polynucleotide. In a preferred aspect of the invention these polypeptides retain the same biological action as the polypeptide encoded by the reference polynucleotide.

The polynucleotide of this invention was recovered from a genomic gene library from *Thermococcus alcaliphihes* AEDII12RA. A gene library was generated and excisions were performed according to the protocols/methods hereinafter described.

The polynucleotide of the present invention may be in the form of RNA or DNA, which DNA includes cDNA, genomic DNA, and synthetic DNA. The DNA may be double-stranded or single-stranded, and if single stranded may be the coding strand or non-coding (anti-sense) strand. The coding sequence which encodes the mature enzyme may be identical to the coding sequence shown in Figure 1 (SEQ ID NO:3) or may be a different coding sequence, which coding sequence, as a result of the redundancy or degeneracy of the genetic code, encodes the same mature enzyme as the DNA of Figure 1 (SEQ ID NO:3).

The polynucleotides which encode for the mature enzyme of Figure 1 (SEQ ID NO:4) may include, but are not limited to: only the coding sequence for the mature enzyme; the coding sequence for the mature enzyme and additional coding sequence such as a leader sequence or a proprotein sequence; the coding sequence for the mature enzyme (and optionally additional coding sequence) and non-coding sequence, such as introns or non-coding sequence 5' and/or 3' of the coding sequence for the mature enzyme.

Thus, the term "polynucleotide encoding an enzyme (protein)" encompasses a polynucleotide which includes only coding sequence for the enzyme as well as a polynucleotide which includes additional coding and/or non-coding sequence(s).

The present invention further relates to variants of the herein described polynucleotide which code for fragments, analogs and derivatives of the enzyme having

the deduced amino acid sequence of Figure 1 (SEQ ID NO:4). The variant of the polynucleotide may be a naturally occurring or a non-naturally occurring variant of the polynucleotide.

Thus, the present invention includes polynucleotides encoding the same mature enzyme as shown in Figure 1 (SEQ ID NO:4) as well as variants of such polynucleotides which variants code for a fragment, derivative or analog of the enzyme of Figure 1 (SEQ ID NO:4). Such nucleotide variants include deletion variants, substitution variants and addition or insertion variants.

As indicated herein, the polynucleotide may have a coding sequence which is a naturally occurring allelic variant of the coding sequence shown in Figure 1 (SEQ ID NO:3). As known in the art, an allelic variant is an alternate form of a polynucleotide sequence which may have a substitution, deletion or addition of one or more nucleotides, which does not substantially alter the function of the encoded protein.

Fragments of the full length gene of the present invention may be used as hybridization probes for a cDNA or a genomic library to isolate the full length DNA and to isolate other DNAs which have a high sequence similarity to the gene or similar biological activity. Probes of this type preferably have at least 10, preferably at least 15, and even more preferably at least 30 bases and may contain, for example, at least 50 or more bases. The probe may also be used to identify a DNA clone corresponding to a full length transcript and a genomic clone or clones that contain the complete gene including regulatory and promotor regions, exons and introns. An example of a screen comprises isolating the coding region of the gene by using the known DNA sequence to synthesize an oligonucleotide probe. Labeled oligonucleotides having a sequence complementary to that of the gene of the present invention are used to screen a library of genomic DNA to identify members of the library to which the probe hybridizes.

It is also appreciated that such probes can be and are preferably labeled with an analytically detectable reagent to facilitate identification of the probe. Useful reagents include but are not limited to radioactive isotopes, fluorescent dyes or enzymes capable of catalyzing the formation of a detectable product. The probes are thus useful to isolate complementary copies of DNA from other sources or to screen such sources for related sequences.

The present invention further relates to polynucleotides which hybridize to the hereinabove-described sequence if there is at least 70%, preferably at least 90%, and more preferably at least 95% identity between the sequences. The present invention particularly relates to polynucleotides which hybridize under stringent conditions to the above-described polynucleotide. As herein used, the term "stringent conditions" means hybridization will occur only if there is at least 95% and preferably at least 97% identity between the sequences. The polynucleotides which hybridize to the hereinabove described polynucleotide in a preferred embodiment encode enzymes which either retain substantially the same biological function or activity as the mature enzyme encoded by the DNA of Figure 1 (SEQ ID NO:3).

Alternatively, the polynucleotide may have at least 15 bases, preferably at least 30 bases, and more preferably at least 50 bases which hybridize to any part of a polynucleotide of the present invention and which has an identity thereto, as hereinabove described, and which may or may not retain activity. For example, such polynucleotides may be employed as probes for the polynucleotide of SEQ ID NO:3, for example, for recovery of the polynucleotide or as a diagnostic probe or as a PCR primer.

Thus, the present invention is directed to polynucleotides having at least a 70% identity, preferably at least 90% identity and more preferably at least a 95% identity to a polynucleotide which encodes the enzyme of SEQ ID NO:4 as well as fragments thereof, which fragments have at least 15 bases, preferably at least 30 bases and most

preferably at least 50 bases, which fragments are at least 90% identical, preferably at least 95% identical and most preferably at least 97% identical under stringent conditions to any portion of a polynucleotide of the present invention.

The present invention further relates to an enzyme which has the deduced amino acid sequence of Figure 1 (SEQ ID NO:4) as well as fragments, analogs and derivatives thereof.

The terms "fragment," "derivative" and "analog" when referring to the enzyme of Figure 1 (SEQ ID NO:4) mean enzymes which retain essentially the same biological function or activity as the enzyme of SEQ ID NO:4. Thus, an analog includes a proprotein which can be activated by cleavage of the proprotein portion to produce an active mature enzyme.

The enzyme of the present invention may be a recombinant enzyme, a natural enzyme or a synthetic enzyme, preferably a recombinant enzyme.

The fragment, derivative or analog of the enzyme of Figure 1 (SEQ ID NO:4) may be (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code, or (ii) one in which one or more of the amino acid residues includes a substituent group, or (iii) one in which the mature enzyme is fused with another compound, such as a compound to increase the half-life of the enzyme (for example, polyethylene glycol), or (iv) one in which the additional amino acids are fused to the mature enzyme, such as a leader or secretory sequence or a sequence which is employed for purification of the mature enzyme or a proprotein sequence. Such fragments, derivatives and analogs are deemed to be within the scope of those skilled in the art from the teachings herein.

The enzyme and polynucleotide of the present invention are preferably provided in an isolated form, and preferably are purified to homogeneity. The term "isolated" means that the material is removed from its original environment (*e.g.*, the natural environment if it is naturally occurring). For example, a naturally-occurring polynucleotide or enzyme present in a living animal is not isolated, but the same polynucleotide or enzyme, separated from some or all of the coexisting materials in the natural system, is isolated. Such polynucleotides could be part of a vector and/or such polynucleotides or enzymes could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment.

The enzymes of the present invention include the enzyme of SEQ ID NO:4 (in particular the mature enzyme) as well as enzymes which have at least 70% similarity (preferably at least 70% identity) to the enzyme of SEQ ID NO:4 and more preferably at least 90% similarity (more preferably at least 90% identity) to the enzyme of SEQ ID NO:4 and still more preferably at least 95% similarity (still more preferably at least 95% identity) to the enzyme of SEQ ID NO:4 and also include portions of such enzymes with such portion of the enzyme generally containing at least 30 amino acids and more preferably at least 50 amino acids.

As known in the art "similarity" between two enzymes is determined by comparing the amino acid sequence and its conserved amino acid substitutes of one enzyme to the sequence of a second enzyme.

A variant, *i.e.* a "fragment", "analog" or "derivative" polypeptide, and reference polypeptide may differ in amino acid sequence by one or more substitutions, additions, deletions, fusions and truncations, which may be present in any combination. Among preferred variants are those that vary from a reference by conservative amino acid substitutions. Such substitutions are those that substitute a given amino acid in a polypeptide by another amino acid of like characteristics. Typically seen as conservative substitutions are the replacements, one for another, among the aliphatic amino acids Ala,

Val, Leu and Ile; interchange of the hydroxyl residues Ser and Thr, exchange of the acidic residues Asp and Glu, substitution between the amide residues Asn and Gln, exchange of the basic residues Lys and Arg and replacements among the aromatic residues Phe, Tyr. Most highly preferred are variants which retain the same biological function and activity as the reference polypeptide from which it varies.

Fragments or portions of the enzymes of the present invention may be employed for producing the corresponding full-length enzyme by peptide synthesis; therefore, the fragments may be employed as intermediates for producing the full-length enzymes. Fragments or portions of the polynucleotides of the present invention may be used to synthesize full-length polynucleotides of the present invention.

The present invention also relates to vectors which include polynucleotides of the present invention, host cells which are genetically engineered with vectors of the invention and the production of enzymes of the invention by recombinant techniques.

Host cells are genetically engineered (transduced or transformed or transfected) with the vectors of this invention which may be, for example, a cloning vector or an expression vector. The vector may be, for example, in the form of a plasmid, a viral particle, a phage, *etc.* The engineered host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants or amplifying the genes of the present invention. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

The polynucleotides of the present invention may be employed for producing enzymes by recombinant techniques. Thus, for example, the polynucleotide may be included in any one of a variety of expression vectors for expressing an enzyme. Such vectors include chromosomal, nonchromosomal and synthetic DNA sequences, *e.g.*, derivatives of SV40; bacterial plasmids; phage DNA; baculovirus; yeast plasmids;

vectors derived from combinations of plasmids and phage DNA, viral DNA such as vaccinia, adenovirus, fowl pox virus, and pseudorabies. However, any other vector may be used as long as it is replicable and viable in the host.

The appropriate DNA sequence may be inserted into the vector by a variety of procedures. In general, the DNA sequence is inserted into an appropriate restriction endonuclease site(s) by procedures known in the art. Such procedures and others are deemed to be within the scope of those skilled in the art.

The DNA sequence in the expression vector is operatively linked to an appropriate expression control sequence(s) (promoter) to direct mRNA synthesis. As representative examples of such promoters, there may be mentioned: LTR or SV40 promoter, the *E. coli. lac* or *trp*, the phage lambda P_L promoter and other promoters known to control expression of genes in prokaryotic or eukaryotic cells or their viruses. The expression vector also contains a ribosome binding site for translation initiation and a transcription terminator. The vector may also include appropriate sequences for amplifying expression.

In addition, the expression vectors preferably contain one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells such as dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, or such as tetracycline or ampicillin resistance in *E. coli*.

The vector containing the appropriate DNA sequence as hereinabove described, as well as an appropriate promoter or control sequence, may be employed to transform an appropriate host to permit the host to express the protein.

As representative examples of appropriate hosts, there may be mentioned: bacterial cells, such as *E. coli, Streptomyces, Bacillus subtilis*; fungal cells, such as yeast; insect cells such as *Drosophila S2* and *Spodoptera Sf9*; animal cells such as CHO,

COS or Bowes melanoma; adenoviruses; plant cells, *etc.* The selection of an appropriate host is deemed to be within the scope of those skilled in the art from the teachings herein.

More particularly, the present invention also includes recombinant constructs comprising one or more of the sequences as broadly described above. The constructs comprise a vector, such as a plasmid or viral vector, into which a sequence of the invention has been inserted, in a forward or reverse orientation. In a preferred aspect of this embodiment, the construct further comprises regulatory sequences, including, for example, a promoter, operably linked to the sequence. Large numbers of suitable vectors and promoters are known to those of skill in the art, and are commercially available. The following vectors are provided by way of example; Bacterial: pQE70, pQE60, pQE-9 (Qiagen), pBluescript II KS (Stratagene), ptrc99a, pKK223-3, pDR540, pRIT2T (Pharmacia); Eukaryotic: pXT1, pSG5 (Stratagene) pSVK3, pBPV, pMSG, pSVL, SV40 (Pharmacia). However, any other plasmid or vector may be used as long as they are replicable and viable in the host.

Promoter regions can be selected from any desired gene using CAT (chloramphenicol transferase) vectors or other vectors with selectable markers. Two appropriate vectors are pKK232-8 and pCM7. Particular named bacterial promoters include lacI, lacZ, T3, T7, gpt, lambda P_R, P_L and trp. Eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retrovirus, and mouse metallothionein-I. Selection of the appropriate vector and promoter is well within the level of ordinary skill in the art.

In a further embodiment, the present invention relates to host cells containing the above-described constructs. The host cell can be a higher eukaryotic cell, such as a mammalian cell, or a lower eukaryotic cell, such as a yeast cell, or the host cell can be a prokaryotic cell, such as a bacterial cell. Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-Dextran mediated transfection,

or electroporation (Davis, L., Dibner, M., Battey, I., *Basic Methods in Molecular Biology*, (1986)).

The constructs in host cells can be used in a conventional manner to produce the gene product encoded by the recombinant sequence. Alternatively, the enzyme of the invention can be synthetically produced by conventional peptide synthesizers.

Mature proteins can be expressed in mammalian cells, yeast, bacteria, or other cells under the control of appropriate promoters. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention. Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described by Sambrook *et al., Molecular Cloning: A Laboratory Manual, Second Edition*, Cold Spring Harbor, N.Y., (1989), the disclosure of which is hereby incorporated by reference.

Transcription of the DNA encoding the enzymes of the present invention by higher eukaryotes is increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp that act on a promoter to increase its transcription. Examples include the SV40 enhancer on the late side of the replication origin bp 100 to 270, a cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

Generally, recombinant expression vectors will include origins of replication and selectable markers permitting transformation of the host cell, *e.g.*, the ampicillin resistance gene of *E. coli* and *S. cerevisiae* TRP1 gene, and a promoter derived from a highly-expressed gene to direct transcription of a downstream structural sequence. Such promoters can be derived from operons encoding glycolytic enzymes such as 3-phosphoglycerate kinase (PGK), α-factor, acid phosphatase, or heat shock proteins, among others. The heterologous structural sequence is assembled in appropriate phase

with translation initiation and termination sequences, and preferably, a leader sequence capable of directing secretion of translated enzyme. Optionally, the heterologous sequence can encode a fusion enzyme including an N-terminal identification peptide imparting desired characteristics, *e.g.*, stabilization or simplified purification of expressed recombinant product.

Useful expression vectors for bacterial use are constructed by inserting a structural DNA sequence encoding a desired protein together with suitable translation initiation and termination signals in operable reading phase with a functional promoter. The vector will comprise one or more phenotypic selectable markers and an origin of replication to ensure maintenance of the vector and to, if desirable, provide amplification within the host. Suitable prokaryotic hosts for transformation include *E. coli, Bacillus subtilis, Salmonella typhimurium* and various species within the genera Pseudomonas, Streptomyces, and Staphylococcus, although others may also be employed as a matter of choice.

As a representative but nonlimiting example, useful expression vectors for bacterial use can comprise a selectable marker and bacterial origin of replication derived from commercially available plasmids comprising genetic elements of the well known cloning vector pBR322 (ATCC 37017). Such commercial vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and pGEM1 (Promega Biotec, Madison, WI, USA). These pBR322 "backbone" sections are combined with an appropriate promoter and the structural sequence to be expressed.

Following transformation of a suitable host strain and growth of the host strain to an appropriate cell density, the selected promoter is induced by appropriate means (*e.g.*, temperature shift or chemical induction) and cells are cultured for an additional period.

Cells are typically harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract retained for further purification.

Microbial cells employed in expression of proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents, such methods are well known to those skilled in the art.

Various mammalian cell culture systems can also be employed to express recombinant protein. Examples of mammalian expression systems include the COS-7 lines of monkey kidney fibroblasts, described by Gluzman, *Cell, 23*:175 (1981), and other cell lines capable of expressing a compatible vector, for example, the C127, 3T3, CHO, HeLa and BHK cell lines. Mammalian expression vectors will comprise an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking nontranscribed sequences. DNA sequences derived from the SV40 splice, and polyadenylation sites may be used to provide the required nontranscribed genetic elements.

The enzyme can be recovered and purified from recombinant cell cultures by methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Protein refolding steps can be used, as necessary, in completing configuration of the mature protein. Finally, high performance liquid chromatography (HPLC) can be employed for final purification steps.

The enzyme of the present invention may be a naturally purified product, or a product of chemical synthetic procedures, or produced by recombinant techniques from a prokaryotic or eukaryotic host (for example, by bacterial, yeast, higher plant, insect and mammalian cells in culture). Depending upon the host employed in a recombinant

production procedure, the enzymes of the present invention may be glycosylated or may be non-glycosylated. Enzymes of the invention may or may not also include an initial methionine amino acid residue.

The polynucleotide of this invention was recovered from a genomic gene library from *Thermococcus alcaliphilus* AEDII12RA. The gene library was generated in the λZAP2 cloning vector (Stratagene). Mass excisions were performed on these libraries to generate libraries in the pBluescript phagemid. Libraries were generated and excisions were performed according to the protocols/methods hereinafter described.

Example 1

Production of the Expression Gene Bank

Colonies containing pBluescript plasmids with random inserts from the organism *Thermococcus alcaliphilus* AEDII12RA were obtained from an original λZAP2 genomic library generated according to the manufacturer's (Stratagene) protocol. The clones were then excised from λZAP2 to pBluescript. The clones were excised to pBluescript according to the method of Hay and Short. (Hay, B. and Short, J. *Strategies*, **1992**, 5:16.) The resulting colonies were picked with sterile toothpicks and used to singly inoculate each of the wells of 96-well microtiter plates. The wells contained 250 μL of LB media with 100 μg/ml methicillin, and 10% v/v glycerol (LB Amp/Meth, glycerol). The cells were grown overnight at 37°C without shaking. This constituted generation of the "Source GeneBank"; each well of the Source GeneBank thus contained a stock culture of *E. coli* cells, each of which contained a pBluescript plasmid with a unique DNA insert.

Example 2

Screening for Glycosidase Activity

The plates of the Source GeneBank were used to multiply inoculate a single plate (the "Condensed Plate") containing in each well 200 μL of LB Amp/Meth, glycerol.

This step was performed using the High Density Replicating Tool (HDRT) of the Beckman Biomek with a 1% bleach, water, isopropanol, air-dry sterilization cycle in between each inoculation. Each well of the Condensed Plate thus contained 10 to 12 different pBluescript clones from each of the source library plates. The Condensed Plate was grown for 16h at 37°C and then used to inoculate two white 96-well Polyfiltronics microtiter daughter plates containing in each well 250 μL of LB Amp/Meth (without glycerol). The original condensed plate was put in storage -80°C. The two condensed daughter plates were incubated at 37°C for 18 h.

A '600 μM substrate stock solution' was prepared as follows: 25 mg of each of four compounds was dissolved in the appropriate volume of DMSO to yield a 25.2 mM solution. The compounds used were 4-methylumbelliferyl β-D-xyloside, 4-methylumbelliferyl α-D-galactoside, 4-methylumbelliferyl α-D-mannopyranoside, and 4-methylumbelliferyl β-D-mannopyranoside. Two hundred fifty microliters of each DMSO solution was added to ca. 9 mL of 50 mM, pH 7.5 Hepes buffer. The volume was taken to 10.5 mL with the above Hepes buffer to yield a clear solution. All four umbelliferones were obtained from Sigma Chemical Co.

Fifty μL of the '600 μM stock solution' was added to each of the wells of a white condensed plate using the Biomek to yield a final concentration of substrate of $\sim$100 μM. The fluorescence values were recorded (excitation = 326 nm, emission = 450 nm) on a plate reading fluorometer immediately after addition of the substrate. The plate was incubated at 70°C for 60 min and the fluorescence values were recorded again. The initial and final fluorescence values were subtracted to determine if an active clone was present by an increase in fluorescence over the majority of the other wells.

Example 3

Isolation of Active Clone and Substrate Specificity Determination

In order to isolate the individual clone which carried the activity, the Source GeneBank plates were thawed and the individual wells used to singly inoculate a new plate containing LB/Amp/Meth. As above the plate was incubated at 37°C to grow the cells, the 50 μL of 600 μM substrate stock solution added using the Biomek. Once the active well from the source plate was identified, the cells from the source plate were streaked on agar with LB/Amp/Meth and grown overnight at to 37°C to obtain single colonies. Eight single colonies were picked with a sterile toothpick and used to singly inoculate the wells of a 96-well microtiter plate. The wells contained 250 μL of LB/Amp/Meth. The cells were grown overnight at 37°C without shaking. A 200 μL aliquot was removed from each well and assayed with the substrates as above. The most active clone was identified and the remaining 50 μL of culture was used to streak an agar plate with LB/Amp/Meth. Eight single colonies were picked, grown and assayed as above. The most active clone was used to inoculate 3mL cultures of LB/Amp/Meth, which were grown overnight. The plasmid DNA was isolated from the cultures and utilized for sequencing. Colonies from this final streak onto the agar plate were also used to inoculate wells containing 250 μL of LB/Amp/Meth. In addition, colonies containing plasmids with no inserts were used as negative controls. A 600 μM solution of each individual substrate was made up for the purpose of determining the substrate specificity of the enzyme. Fifty μL of each of the four substrates were added individually to the test and control wells and assayed for activity as above. Only the wells which contained the 4-methylumbelliferyl α-D-galactoside showed an increase in fluorescence indicating activity.

Numerous modifications and variations of the present invention are possible in light of the above teachings and, therefore, within the scope of the appended claims, the invention may be practiced otherwise than as particularly described.

PATENT
Attorney Docket No.: DIVER1120-1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Dennis Murphy, et al.

Parent Application No.: 08/613,220
Parent Filed: March 8, 1996

Application No.: Unknown
Filed: September 28, 1999

For: ALPHA-GALACTOSIDASE

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) Examiner:
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Assistant Commissioner for Patents
Washington, D.C. 20231

PERMISSION TO USE SEQUENCE LISTING

Sir:

The above-identified patent application lacks a substitute paper copy of the Sequence Listing for inclusion into the Specification, as well as a computer readable form of the Sequence Listing. Applicants respectfully direct the attention of the Office to the following:

1. A complete paper copy of the Sequence Listing is to replace the existing Sequence Listing and is to be inserted into the Specification beginning at page 29 and being numbered consecutively thereafter. This paper copy is identical to the paper copy of the Sequence Listing which is included in the Specification of the parent priority patent; to wit, U.S. Patent Application Serial No. 08/613,220 (the "'220 application").
2. A computer-readable form of the Sequence Listing identical to the paper copy of the Sequence Listing is on file in the '220 application.

Pursuant to 37 CFR §1.821(e) I, reference to the computer-readable form on file in the '220 application is hereby made, which computer-readable form was filed in the '220 application on April 30, 1996. Said computer-readable form is identical to both the paper copy of the Sequence Listing on file in the '220 application and the paper copy of the Sequence Listing submitted in the present application.

Applicants submit that the foregoing satisfies the requirements of Rule §1.821. If there are any questions regarding this response, the Office is invited to contact the undersigned.

It is understood that this perfects the application and no additional papers or filing fees are required. If there are any other charges, or any credits, please apply them to Deposit Account 07-1895.

Respectfully submitted,

Dated: September 28, 1999

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: MURPHY, Dennis
REID, John

(ii) TITLE OF INVENTION: Alpha Glycosidase

(iii) NUMBER OF SEQUENCES: 4

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: CARELLA, BYRNE, BAIN, GILFILLAN, CECCHI, STEWART & OLSTEIN
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(C) CITY: ROSELAND
(D) STATE: NEW JERSEY
(E) COUNTRY: USA
(F) ZIP: 07068

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: 3.5 INCH DISKETTE
(B) COMPUTER: IBM PS/2
(C) OPERATING SYSTEM: MS-DOS
(D) SOFTWARE: ASCII

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: Unassigned
(B) FILING DATE: Concurrently
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
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(B) REGISTRATION NUMBER: 28,019
(C) REFERENCE/DOCKET NUMBER: 331400-40

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 201-894-1700
(B) TELEFAX: 201-994-1744

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 52 NUCLEOTIDES
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: SINGLE
(D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CCGAGAATTC ATTAAAGAGG AGAAATTAAC TATGAGAGCG CTCGTCTTTC AC 52

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 31 NUCLEOTIDES
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: SINGLE
(D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CGGAAGATCT AGGTTCCCCA TTTTCACCCC T 31

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 1,095 NUCLEOTIDES
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: SINGLE
(D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
TTG AGA GCG CTC GTC TTT CAC GGC AAC CTC CAG TAT GCC GAA ATC CCA      48
Leu Arg Ala Leu Val Phe His Gly Asn Leu Gln Tyr Ala Glu Ile Pro
                  5                  10                  15

AAG AGC GAA ATC CCA AAG GTC ATA GAG AAG GCA TAC ATC CCA GTC ATC      96
Lys Ser Glu Ile Pro Lys Val Ile Glu Lys Ala Tyr Ile Pro Val Ile
             20                  25                  30

GAG ACA CTG ATT AAA GAA GAA ATT CCT TTT GGG CTC AAC ATA ACG GGC     144
Glu Thr Leu Ile Lys Glu Glu Ile Pro Phe Gly Leu Asn Ile Thr Gly
         35                  40                  45

TAT ACC TTA AAG TTC CTC CCG AAG GAT ATT ATA GAC CTC GTT AAA GGG     192
Tyr Thr Leu Lys Phe Leu Pro Lys Asp Ile Ile Asp Leu Val Lys Gly
     50                  55                  60

GGC ATC GCG AGT GAC CTG ATA GAG ATA ATC GGA ACG AGC TAC ACG CAC     240
Gly Ile Ala Ser Asp Leu Ile Glu Ile Ile Gly Thr Ser Tyr Thr His
 65                  70                  75                  80

GCA ATA CTC CCC CTC CTC CCG CTT AGC AGA GTA GAA GCA CAA GTT CAG     288
Ala Ile Leu Pro Leu Leu Pro Leu Ser Arg Val Glu Ala Gln Val Gln
                 85                  90                  95

AGA GAT AGG GAA GTT AAG GAA GAG CTC TTC GAG CTT TCT CCA AAG GGA     336
Arg Asp Arg Glu Val Lys Glu Glu Leu Phe Glu Val Ser Pro Lys Gly
            100                 105                 110
```

```
TTC TGG CTG CCA GAG CTC GCC TAT GAC CCG ATA ATC CCT GCC ATA CTG      384
Phe Trp Leu Pro Glu Leu Ala Tyr Asp Pro Ile Ile Pro Ala Ile Leu
        115                 120                 125

AAG GAC AAC GGT TAT GAG TAT CTA TTC GCC GAC GGG GAG GCG ATG CTT      432
Lys Asp Asn Gly Tyr Glu Tyr Leu Phe Ala Asp Gly Glu Ala Met Leu
    130                 135                 140

TTC TCA GCT CAT CTC AAC TCG GCG ATA AAG CCA ATT AAA CCG CTC TAT      480
Phe Ser Ala His Leu Asn Ser Ala Ile Lys Pro Ile Lys Pro Leu Tyr
145                 150                 155                 160

CCA CAC CTT ATA AAG GCC CAA AGG GAA AAG CGC TTT AGG TAC ATC AGC      528
Pro His 3Leu Ile Lys Ala Gln Arg Glu Lys Arg Phe Arg Tyr Ile Ser
                165                 170                 175

TAT CTC CTT GGT CTC AGG GAG CTT AGG AAG GCG ATA AAG CTC GTT TTT      576
Tyr Leu Leu Gly Leu Arg Glu Leu Arg Lys Ala Ile Lys Leu Val Phe
            180                 185                 190

GAA GGT AAG GTA ACG CTA AAG GCA GTC AAA GAC ATC GAA GCC GTA CCC      624
Glu Gly Lys Val Thr Leu Lys Ala Val Lys Asp Ile Glu Ala Val Pro
        195                 200                 205

GTT TGG GTG GCC GTG AAC ACG GCT GTA ATG CTC GGC ATC GGA AGG CTT      672
Val Trp Val Ala Val Asn Thr Ala Val Met Leu Gly Ile Gly Arg Leu
    210                 215                 220

CCT CTT ATG AAT CCT AAG AAA GTG GCG AGC TGG ATA GAG GAC AAG GAC      720
Pro Leu Met Asn Pro Lys Lys Val Ala Ser Trp Ile Glu Asp Lys Asp
225                 230                 235                 240

AAC ATT CTT CTA TAC GGC ACC GAT ATA GAG TTC ATT GGC TAT AGG GAC      768
Asn Ile Leu Leu Tyr Gly Thr Asp Ile Glu Phe Ile Gly Tyr Arg Asp
                245                 250                 255

ATT GCA GGC TAC AGA ATG AGT GTT GAG GGA TTA TTA GAG GTT ATA GAC      816
Ile Ala Gly Tyr Arg Met Ser Val Glu Gly Leu Leu Glu Val Ile Asp
            260                 265                 270

GAG CTC AAC TCG GAA CTG TGC CTT CCC TCA GAG CTG AAG CAC AGT GGA      864
Glu Leu Asn Ser Glu Leu Cys Leu Pro Ser Glu Leu Lys His Ser Gly
        275                 280                 285

AGG GAG CTC TAC TTA CGG ACT TCG AGT TGG GCA CCA GAT AAG AGC TTG      912
Arg Glu Leu Tyr Leu Arg Thr Ser Ser Trp Ala Pro Asp Lys Ser Leu
    290                 295                 300
```

```
AGG ATA TGG AGA GAG   C GAA GGG AAC GCA AGA CTT AAT ATG CTG TCC      960
Arg Ile Trp Arg Glu   p Glu Gly Asn Ala Arg Leu Asn M   Leu Ser
305                 310                 315                 320

TAC AAT ATG AGG GGC GAA CTC GCC CTT TTA GCC GAG AAC AGC GAT GCA    1008
Tyr Asn Met Arg Gly Glu Leu Ala Phe Leu Ala Glu Asn Ser Asp Ala
                325                 330                 335

AGG GGA TGG GAG CCC CTC CCT GAG AGG AGG CTG GAT GCC TTC CGG GCG    1047
Arg Gly Trp Glu Pro Leu Pro Glu Arg Arg Leu Asp Ala Phe Arg Ala
            340                 345                 350

ATA TAT AAC GAT TGG AGG GGT GAA AAT GGG GAA CCT TAG                1086
Ile Tyr Asn Asp Trp Arg Gly Glu Asn Gly Glu Pro End
        355                 360                 365
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 364 AMINO ACIDS
(B) TYPE: POLYPEPTIDE
(D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: PROTEIN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Leu Arg Ala Leu Val Phe Mis Gly Asn Leu Gln Tyr Ala Glu Ile
  5                  10                  15

Phe Lys Ser Glu Ile Pro Lys Val Ile Glu Lys Ala Tyr Ile Pro
              20                  25                  30

Val Ile Glu Thr Leu Ile Lys Glu Gln Ile Pro Phe Gly Leu Asn
          35                  40                  45

Ile Thr Gly Tyr Thr Leu Lys Phe Leu Pro Lys Asn Ile Ile Asp
      50                  55                  60

Leu Val Lys Gly Gly Ile Ala Ser Asp Leu Ile Glu Ile Ile Gly
  65                  70                  75

Thr Ser Tyr Tyr His Ala Ile Leu Pro Leu Leu Pro Leu Ser Arg
              80                  85                  90

Val Glu Ala Glu Val Gln Arg Asp Arg Glu Val Lys Glu Glu Leu
          95                  100                 105

Phe Glu Val Ser Pro Lys Gly Phe Trp Leu Pro Glu Leu Ala Tyr
      110                 115                 120

Asn Pro Ile Ile Phe Ala Ile Leu Lys Asp Asn Gly Tyr Glu Tyr
  125                 130                 135

Leu Phe Ala Asp Gly Glu Ala Met Leu Phe Ser Ala His Leu Asn
              140                 145                 150
```

Ser Ala Ile Lys Pro Ile Lys Pro Leu Tyr Pro His Leu Ile Lys
155 160 165

Ala Gln Arg Glu Lys Arg Phe Arg Tyr Ile Ser Tyr Leu Leu GLy
170 175 180

Leu Arg Glu Leu Arg Lys Ala Ile Lys Leu Val Phe Glu Gly Lys
185 190 195

Val Thr Leu Lys Ala Val Lys Asp Ile Glu Ala Val Pro Val Trp
200 205 210

Val Ala Val Asn Thr Ala Val Met Leu Gly Ile Gly Arg Leu Pro
215 220 225

Leu Met As? Pro Lys Lys Val Ala Ser Trp Ile Glu Asp Lys Asp
230 235 240

Asn Ile Leu Leu Tyr Gly Thr Asp Ile Glu Phe Ile Gly Tyr Arg
245 250 255

Asp Ile Ala Gly Tyr Arg Met Ser Val Glu Gly Leu Leu Glu Val
260 265 270

Ile Asp Glu Leu Asn Ser Glu Leu Cys Leu Pro Ser Glu Leu Lys
275 280 285

His Ser Gly Arg Glu Leu Tyr Leu Arg Thr Ser Ser Trp Ala Pro
290 295 300

Asp Lys Ser Leu Arg Ile Trp Arg Glu Asp Glu Gly Asn Ala Arg
305 310 315

Leu Asn Met Leu Ser Tyr Asn Met Arg Gly Glu Leu Ala Phe Leu
320 325 330

Ala Glu Asn Ser Asp Ala Arg Gly Trp Glu Pro Leu Pro Gln Arg
335 340 345

Arg Leu Asp Ala Phe Arg Ala Ile Tyr Asn Asp Trp Arg Gly Glu
350 355 360

Asn Gly Glu Pro

WHAT IS CLAIMED IS:

1. An isolated polynucleotide comprising a member selected from the group consisting of:

(a) a polynucleotide having at least a 70% identity to a polynucleotide encoding an enzyme comprising amino acid sequences set forth in SEQ ID NO:4;

(b) a polynucleotide which is complementary to the polynucleotide of (a); and

(c) a polynucleotide comprising at least 15 bases of the polynucleotide of (a) or (b).

2. The polynucleotide of Claim 1 wherein the polynucleotide is DNA.

3. The polynucleotide of Claim 1 wherein the polynucleotide is RNA.

4. The polynucleotide of Claim 2 which encodes an enzyme comprising amino acids 1 to 364 of SEQ ID NO:4.

5. An isolated polynucleotide comprising a member selected from the group consisting of:

(a) a polynucleotide having at least a 70% identity to a polynucleotide encoding an enzyme encoded by the DNA contained in ATCC Deposit No. _____, wherein said enzyme is AEDII12RA-α-gal-18GC;

(b) a polynucleotide complementary to the polynucleotide of (a); and

(c) a polynucleotide comprising at least 15 bases of the polynucleotide of (a) and (b).

6. A vector comprising the DNA of Claim 2.

7. A host cell comprising the vector of Claim 13.

8. A process for producing a polypeptide comprising: expressing from the host cell of Claim 14 a polypeptide encoded by said DNA.

9. A process for producing a cell comprising: transforming or transfecting the cell with the vector of Claim 13 such that the cell expresses the polypeptide encoded by the DNA contained in the vector.

10. An enzyme comprising a member selected from the group consisting of:

(a) an enzyme comprising an amino acid sequence which is at least 70% identical to the amino acid sequence set forth in SEQ ID NO:4; and

(b) an enzyme which comprises at least 30 amino acid residues to the enzyme of (a).

11. An enzyme comprising a member selected from the group consisting of:

(a) an enzyme comprising an amino acid sequence as set forth in in SEQ ID NO:4; and

(b) an enzyme which comprises at least 30 amino acid residues to the enzyme of (a).

12. A method for hydrolyzing α-galactose bonds comprising:

administering an effective amount of an enyzme having the amino acid sequence set forth in SEQ ID NO:4.

ABSTRACT

A thermostable alpha-glycosidase derived from various *Thermococcus, alcaliphilus AEDII12RA* is disclosed. The enzymes are produced from native or recombinant host cells and can be utilized in the food processing industry.

FIG. 1

```
TTG AGA GCG CTC GTC TTT CAC GGC AAC CTC CAG TAT GCC GAA ATC CCA  48
Leu Arg Ala Leu Val Phe His Gly Asn Leu Gln Tyr Ala Glu Ile Pro
                5                  10                  15

AAG AGC GAA ATC CCA AAG GTC ATA GAG AAG GCA TAC ATC CCA GTC ATC  96
Lys Ser Glu Ile Pro Lys Val Ile Glu Lys Ala Tyr Ile Pro Val Ile
            20                  25                  30

GAG ACA CTG ATT AAA GAA GAA ATT CCT TTT GGG CTC AAC ATA ACG GGC 144
Glu Thr Leu Ile Lys Glu Glu Ile Pro Phe Gly Leu Asn Ile Thr Gly
        35                  40                  45

TAT ACC TTA AAG TTC CTC CCG AAG GAT ATT ATA GAC CTC GTT AAA GGG 192
Tyr Thr Leu Lys Phe Leu Pro Lys Asp Ile Ile Asp Leu Val Lys Gly
    50                  55                  60

GGC ATC GCG AGT GAC CTG ATA GAG ATA ATC GGA ACG AGC TAC ACG CAC 240
Gly Ile Ala Ser Asp Leu Ile Glu Ile Ile Gly Thr Ser Tyr Thr His
65                  70                  75                  80

GCA ATA CTC CCC CTC CTC CCG CTT AGC AGA GTA GAA GCA CAA GTT CAG 288
Ala Ile Leu Pro Leu Leu Pro Leu Ser Arg Val Glu Ala Gln Val Gln
                85                  90                  95

AGA GAT AGG GAA GTT AAG GAA GAG CTC TTC GAG CTT TCT CCA AAG GGA 336
Arg Asp Arg Glu Val Lys Glu Glu Leu Phe Glu Val Ser Pro Lys Gly
            100                 105                 110

TTC TGG CTG CCA GAG CTC GCC TAT GAC CCG ATA ATC CCT GCC ATA CTG 384
Phe Trp Leu Pro Glu Leu Ala Tyr Asp Pro Ile Ile Pro Ala Ile Leu
        115                 120                 125
```

FIG. 1A

```
AAG GAC AAC GGT TAT GAG TAT CTA TTC GCC GAC GGG GAG GCG ATG CTT   432
Lys Asp Asn Gly Tyr Glu Tyr Leu Phe Ala Asp Gly Glu Ala Met Leu
    130             135                 140

TTC TCA GCT CAT CTC AAC TCG GCG ATA AAG CCA ATT AAA CCG CTC TAT   480
Phe Ser Ala His Leu Asn Ser Ala Ile Lys Pro Ile Lys Pro Leu Tyr
145                 150                 155                 160

CCA CAC CTT ATA AAG GCC CAA AGG GAA AAG CGC TTT AGG TAC ATC AGC   528
Pro His 3Leu Ile Lys Ala Gln Arg Glu Lys Arg Phe Arg Tyr Ile Ser
            165                 170                 175

TAT CTC CTT GGT CTC AGG GAG CTT AGG AAG GCG ATA AAG CTC GTT TTT   576
Tyr Leu Leu Gly Leu Arg Glu Leu Arg Lys Ala Ile Lys Leu Val Phe
        180                 185                 190

GAA GGT AAG GTA ACG CTA AAG GCA GTC AAA GAC ATC GAA GCC GTA CCC   624
Glu Gly Lys Val Thr Leu Lys Ala Val Lys Asp Ile Glu Ala Val Pro
    195                 200                 205

GTT TGG GTG GCC GTG AAC ACG GCT GTA ATG CTC GGC ATC GGA AGG CTT   672
Val Trp Val Ala Val Asn Thr Ala Val Met Leu Gly Ile Gly Arg Leu
210                 215                 220

CCT CTT ATG AAT CCT AAG AAA GTG GCG AGC TGG ATA GAG GAC AAG GAC   720
Pro Leu Met Asn Pro Lys Lys Val Ala Ser Trp Ile Glu Asp Lys Asp
225                 230                 235                 240

AAC ATT CTT CTA TAC GGC ACC GAT ATA GAG TTC ATT GGC TAT AGG GAC   768
Asn Ile Leu Leu Tyr Gly Thr Asp Ile Glu Phe Ile Gly Tyr Arg Asp
            245                 250                 255
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FIG. 1B

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ATT GCA GGC TAC AGA ATG AGT GTT GAG GGA TTA TTA GAG GTT ATA GAC  816
Ile Ala Gly Tyr Arg Met Ser Val Glu Gly Leu Leu Glu Val Ile Asp
            260                 265                 270

GAG CTC AAC TCG GAA CTG TGC CTT CCC TCA GAG CTG AAG CAC ATG GGA  864
Glu Leu Asn Ser Glu Leu Cys Leu Pro Ser Glu Leu Lys His Ser Gly
        275                 280                 285

AGG GAG CTC TAC TTA CGG ACT TCG AGT TGG GCA CCA GAT AAG AGC TTG  912
Arg Glu Leu Tyr Leu Arg Thr Ser Ser Trp Ala Pro Asp Lys Ser Leu
    290                 295                 300

AGG ATA TGG AGA GAG GAC GAA GGG AAC GCA AGA CTT AAT ATG CTG TCC  960
Arg Ile Trp Arg Glu Asp Glu Gly Asn Ala Arg Leu Asn Met Leu Ser
305                 310                 315                 320

TAC AAT ATG AGG GGC GAA CTC GCC CTT TTA GCC GAG AAC AGC GAT GCA 1008
Tyr Asn Met Arg Gly Glu Leu Ala Phe Leu Ala Glu Asn Ser Asp Ala
                325                 330                 335

AGG GGA TGG GAG CCC CTC CCT GAG AGG AGG CTG GAT GCC TTC CGG GCG 1047
Arg Gly Trp Glu Pro Leu Pro Glu Arg Arg Leu Asp Ala Phe Arg Ala
            340                 345                 350

ATA TAT AAC GAT TGG AGG GGT GAA AAT GGG GAA CCT TAG             1086
Ile Tyr Asn Asp Trp Arg Gly Glu Asn Gly Glu Pro End
        355                 360                 365
```

PATENT
ATTORNEY DOCKET NO. 09010/0004001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant :	D. Murphy et al.	Art Unit: 1814
Serial No.:	08/613,220	Examiner: K. Hendricks
Filed :	March 8, 1996	
Title :	ALPHA-GALACTOSIDASE	

Assistant Commissioner of Patents
Washington, DC 20231

TRANSMITTAL OF REVOCATION AND NEW POWER OF ATTORNEY

Attached please find an original Revocation and New Power of Attorney from the Assignee relating to this application executed on May 16, 1997. Please substitute the enclosed document for all prior Powers of Attorney.

Please apply any charges to our Deposit Account No. 06-1050.

Respectfully submitted,

Date: 6/13/97

Lisa A. Haile, Ph.D.
Reg. No. 38,347

Fish & Richardson P.C.
4225 Executive Square, Suite 1400
La Jolla, CA 92037

Telephone: 619/678-5070
Facsimile: 619/678-5099

33360

Date of Deposit 6-13-97

I hereby certify under 37 C.F.R. 1.8(a) that this correspondence is being deposited with the United States Postal Service as first class mail with sufficient postage on the date indicated above and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

Stephanie Sharrett

PATENT
ATTORNEY DOCKET NO. 09010/004001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Murphy, D. *et al.*	Art Unit:	1814
Serial No.:	08/613,220	Examiner:	Hendricks, K.
Filed:	3/8/96		
Title:	ALPHA-GALACTOSIDASE		

Assistant Commissioner for Patents
Washington, DC 20231

REVOCATION AND NEW POWER OF ATTORNEY

Under 37 CFR §3.73(b) RECOMBINANT BIOCATALYSIS, INC., a Delaware corporation, certifies that it is the assignee of 100% of the right, title and interest in the patent application identified above by virtue of an assignment from the inventors of the patent application identified above. The assignment was recorded in the Patent and Trademark Office at Reel 8103, Frames 0390-0393, on July 18, 1996.

The undersigned has reviewed all the documents in the chain of title of the patent application identified above and, to the best of undersigned's knowledge and belief, title is in the assignee identified above.

The undersigned, whose title is supplied below, is empowered to act on behalf of the assignee.

Date of Deposit 6-13-97
I hereby certify under 37 CFR 1.8(a) that this correspondence is being deposited with the United States Postal Service as first class mail with sufficient postage on the date indicated above and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

Stephanie Sharrett

Stephanie Sharrett

The undersigned, acting on behalf of the assignee, hereby revokes all powers of attorney previously granted in the application and appoints: John R. Wetherell, Jr., Ph.D. (Reg. No. 31,678); Lisa A. Haile (Reg. No. 38,347); Stacy L. Taylor (Reg. No. 34,842); John Land (Reg. No. 29,554), and June M. Learn (Reg. No. 31,238), of the firm of FISH & RICHARDSON P.C., as its attorneys with full power of substitution and revocation, to prosecute the application and to transact all business in the United States Patent and Trademark Office connected therewith.

Please direct all telephone calls to John R. Wetherell at (619) 678-5070 and all correspondence relative to said application to the following address:

John R. Wetherell, Jr., Ph.D.
FISH & RICHARDSON, P.C.
4225 Executive Square, Suite 1400
La Jolla, California 92037
(619) 678-5099 (Facsimile)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Respectfully submitted,

Date: 5-17-97

Carolyn Erickson
Manager, Business Development and
Regulatory Affairs
RECOMBINANT BIOCATALYSIS, INC.

30888 LJ1

In re Application of
Dennis Murphy, et al.
Filed: September 28, 1999

PATENT
Attorney Docket No. DIVER1120-1

DECLARATION FOR PATENT APPLICATION

As a below-named inventor, I hereby declare that:

My residence, post office address and citizenship is as stated below next to my name.

I believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled ALPHA-GALACTOSIDASE, the specification of which

_____ is attached hereto.

__X__ was filed on September 28, 1999 (Attorney Docket No. DIVER1120-1)

as U.S. Application Serial No. _____

and was amended on _____

if applicable (the "Application").

I hereby authorize and request insertion of the application serial number of the Application when officially known.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability of the subject matter of the Application as defined in Title 37, Code of Federal Regulations ("C.F.R."), § 1.56.

With respect to the Application, I hereby claim the benefit under 35 U.S.C. Section 120 of any United States application(s), or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of the application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. Section 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability of the subject matter of the Application as defined in Title 37, C.F.R., Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of the Application:

08/613,220	March 8, 1996	Issuing: September 28, 1999 as (U.S. Patent No. 5,958,751)
(Application Serial No.)	(Filing Date)	

I hereby claim foreign priority benefits under Title 35, United States Code, § 119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

COUNTRY	APPLICATION NO.	FILING DATE	PRIORITY CLAIMED	
PCT	US97/01452	February 5, 1997	☒Yes	☐ No
			☐ Yes	☐ No

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of first inventor: **Dennis Murphy**

Inventor's signature: _____

Date: _____

Residence: 10 Fairway Road, Paoli, PA 19301

Citizenship: United States

Post Office Address: 10 Fairway Road

Paoli, PA 19301

Full name of second inventor: **John Reid**

Inventor's signature: _____

Date: _____

Residence: 922 Montgomery Ave., Apt. J2; Bryn Mawr, PA 19010

Citizenship: United States

Post Office Address: 922 Montgomery Ave., Apt. J-2

Bryn Mawr, PA 19010

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

ALPHA-GALACTOSIDASE

the specification of which [X] is attached hereto or [ ] was filed on _____ as Application Serial No. _____ and was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with Title 37, Code of Federal Regulations, Section 1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed. Prior Foreign Application(s):

(Number)	(Country)	(Day/Month/Year Filed)	Priority Claimed Yes	No
			☐	☐

I hereby claim the benefit under Title 35, United States Code, Section 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, Section 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, Section 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

(Application Serial No.)	(Filing Date)	(Status - patented, pending, abandoned)
		Pending

I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: John N. Bain (Reg. No. 18,651); John G. Gilfillan, III (Reg. No. 22,746); Elliot M. Olstein (Reg. No. 24,025); Raymond J. Lillie (Reg. No. 31,778); Charles J. Herron (Reg. No. 28,019); William Squire (Reg. No. 25,378); Kenneth S. Weitzman (Reg No. 36,306); and Gregory Ferraro (Reg. No. 36,134). Address correspondence and telephone calls to Charles J. Herron c/o Carella, Byrne, Bain, Gilfillan, Cecchi, Stewart & Olstein, 6 Becker Farm Road, Roseland, NJ 07068 - (201) 994-1700.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of inventor: Dennis Murphy
Inventor's signature: Dennis J. Murphy Date: Mar 5, 1996
Residence: 10 Fairway Road, Paoli, PA 19301 Citizenship: United States
Post Office Address: same

Full name of inventor: John Reid
Inventor's signature: John C. Reid Date: Mar 5 1996
Residence: 922 Montgomery Avenue Apt. J-2, Bryn Mawr, PA 19010 Citizenship: United States
Post Office Address: same